

RESEARCH ARTICLE

Automated and scalable fabrication of scaffolded spheroids as building blocks for modular tissue engineering

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Abstract

Current tissue engineering approaches typically utilize either scaffold-based or scaffold-free strategies. Recently, a third approach has emerged, offering a synergistic method that leverages the benefits of both approaches while avoiding their usual drawbacks. In this strategy, spheroids are formed within highly porous microscaffolds, which subsequently serve as building blocks for larger tissue constructs produced through self-assembly. This study demonstrates the automated fabrication of large numbers of these building blocks, tested in vessels of up to 1,536-well plates. To achieve this, we first developed a microfluidic device capable of sorting highly porous microscaffolds, each 300 µm in diameter, at high flow speeds of up to 300 mm/s. A fluorescent detection system was integrated to identify only intact microscaffolds and deposit them individually into separate wells of cell culture plates. Subsequently, the system automatically dispensed culture medium containing suspended human adipose-derived stem cells into each well. Spheroids were formed within 24 hours of culture with a formation efficiency of 95%. This study presents the development of an automated microfluidic device capable of depositing single microscaffolds and the cells required to form scaffolded spheroids. By enabling large-scale, high-throughput production, this approach advances the third strategy of tissue engineering and supports applications in both screening and clinical translation.

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1. Introduction

Tissue engineering (TE) is an interdisciplinary field combining cells, biomaterials, and biochemical cues, which aims to recreate human tissues *in vitro* with authentic structure and function. These structures can then be used for *in vitro* diagnostic applications or to

restore the functionality of damaged tissue *in vivo*.^{1,2} In the past, two-dimensional cell culture systems predominated, even though they poorly mimicked the *in vivo* niche—a highly complex microenvironment including three-dimensional (3D) biochemical and biomechanical cues. This oversimplification of the intricate tissue microenvironment results in the loss of essential biophysical traits, thereby compromising the performance of TE models. Notably, gene expression and oxygen gradient differences between two-dimensional and 3D models critically influence the cell response and typical cell behavior.³ As a response to these shortcomings, modern 3D cell culture constructs aim to mimic the *in vivo* environment more accurately, providing cell–cell interaction and the physiological cues of the emulated microenvironment.⁴

To date, the formation of engineered tissue constructs has primarily relied on either scaffold-based or scaffold-free approaches.⁵ The scaffold-based approach uses natural or synthetic materials to provide cells with a biodegradable structural support that promotes cellular adhesion and proliferation. It also defines the mechanical properties of the engineered construct and protects cells from external mechanical damage.

Scaffolding materials offer excellent opportunities for TE. However, conventional 3D porous scaffolds, a crucial class of engineered biomaterials, pose challenges such as large seeding variability, especially in deeper regions, and relatively low initial cell densities. Hydrogels and hydrogel-based microparticles, another commonly used group, can directly embed cells, ensuring their even distribution. However, they often have limited mechanical properties, similar to soft tissues, which can be problematic for applications like cartilage or bone TE.⁶ Notably, scaffold-based approaches limit the overall architecture of the engineered construct mostly to the initial shape and size of the biomaterial scaffold prior to cell seeding.⁷ While advanced technologies, such as 3D bioprinting, can fabricate complex tissue architectures, they still face limitations in achieving high cell density and rapid tissue maturation due to bioink constraints and mechanical stresses during printing.⁸ Typical scaffold-free approaches involve the use of cell aggregates (also referred to as spheroids or pellets) and even cell-sheets, which can be stacked to produce building blocks for even larger tissue constructs. Due to the inherent lack of filler material, these strategies enable engineering of artificial tissues at the highest cell densities. Notably, the complete absence of a scaffold can potentially complicate handling and logistics (i.e., long-term viability), leave cells unprotected from mechanical damage, and hamper the dynamic and precise control of the construct's mechanical properties.

A convergent approach was recently introduced as the third strategy of modern TE that builds upon the synergistic advantages of scaffold-based and scaffold-free approaches.⁵ In this approach, spheroids are reinforced through highly porous microscaffolds that act as a protective cage. The so-formed scaffolded spheroids (S-SPHs) then act as building blocks for larger tissues, as they still possess the inherent capability to fuse with neighboring building blocks. Based on the material properties and the design of the microscaffold, the resulting mechanical properties can be controlled accordingly.⁵

The formation of such building blocks requires the presence of a microscaffold during the spheroid formation process. Given the general molecular diffusion limits present in tissue-engineered constructs,⁹ these structures should not exceed 300 μm in diameter. Recently, our group demonstrated that highly porous cage-like microscaffolds, also referred to as buckyballs (BBs) due to the similarity with Buckminster fullerenes, can be used to contain spheroids, forming building blocks capable of fusing via directed self-assembly. However, generating tissue of a physiologically relevant size from S-SPH requires the production of several thousand building blocks, which in turn necessitates seeding anti-adhesive multi-well cell culture plates with precisely one scaffold per well. While the manual deposition of microscaffolds and cells is feasible, it is a labor-intensive process that cannot be scaled indefinitely and does not allow efficient pre-screening of microscaffolds for quality control (i.e., structural integrity).

To reproducibly form uniform S-SPHs, an intact microscaffold must first be placed into a single well, followed by the deposition of a determined number of cells. The process must also be scalable to support the repeated formation of large quantities of these building blocks. Microfluidic devices have previously shown great promise for precise control and manipulation of particles in flow.¹⁰ However, most examples in the literature typically employ microfluidic devices to diverge an inlet flow into two or more collecting channels.¹¹ While this allows continuous physical separation, such systems cannot be used to eject particles individually. Only recently have systems been described to enable the continuous ejection of fluidic droplets from a microfluidic channel.^{12–15} While such devices are useful for the precise ejection of liquids, such as on-demand droplet printing, ejecting particles within such droplets may pose additional challenges. For example, particles are not always positioned in the center of the droplet, making repeatable screening challenging. Furthermore, the sheath air used to form droplets can accelerate one phase over the other, leading to uneven ejection.

In this study, we demonstrate an approach to dispense BB-shaped microscaffolds within a sheath liquid individually and with high precision into microtiter plates using a newly developed microfluidic sorting platform (Figure 1). For performance evaluation of the air pressure-based dispensing method, the intactness of the microscaffold constructs was verified using in-flow fluorescence intensity analysis upstream of the ejector region and was used as a selection criterion quality control measure. Subsequently, automated deposition of human adipose-derived stem cells (hASCs) onto the microscaffolds allowed the formation of S-SPHs already within 24 hours of culture. For the first time, we introduce an automated and reproducible workflow for the mass production of building blocks in the converging TE strategy with various cell culture vessels, including 96-, 384-, and 1,536-well plates.

2. Methodology

2.1. Electromechanical control of the sorting device

A pressure regulator (OB1-MK3, Elveflow, France), as shown in Figure 2, was used to pressurize 50 mL reservoirs containing 400 BBs in 50 mL of deionized water. The BBs were then forwarded to the microfluidic device via polytetrafluoroethylene tubing with an inner diameter of 0.8 mm (Catalog no: 3200068, Blacktrace, Netherlands). The microfluidic device was spaced within the optical path at a precise distance from the lens using a 3D-printed holder attached to the filter cube. The microfluidic valves were

actuated via a three-way valve block (VV061, SMC, Japan), controlled via a USB MUX valve controller (MUX Wire, Elveflow, France). The pressurized air for the valve block was manually set to 3 bar. The pressurized air for ejection was regulated via the Elveflow system and controlled via Microfluidic 3/2 Peek Valve (MPV3/2, Elveflow, France) attached to the MUX valve controller. The well plate was positioned via a motorized microscope stage (SCAN 100 × 100, Märzhäuser Wetzlar, Germany), controlled via a stepper motor controller (TANGO Desktop, Märzhäuser Wetzlar, Germany). All the mechanical and electronic components were controlled via the main Python control program (version 3.8) running on a personal computer.

2.2. Microfabrication

The chip design is shown in Figure 3. The fluidic layer of the dispensing device was produced via soft lithography from 3D-printed molds manufactured using stereolithography from Accura Xtreme (Shapeways, United States). Polydimethylsiloxane (PDMS; Sylgard 184, DowSil, United States) was mixed with the cross-linking agent at a ratio of 10:1; the mixture was spun down for five minutes and then degassed in a vacuum chamber for 15 minutes. Molds were filled with the PDMS mixture and degassed to remove air prior to overnight curing at 70°C. The deflective membrane and the pneumatic layer were produced via xurography.¹⁶ Silicone sheets of 0.25 and 0.5 mm (HT-6240, Rogers Corporation, United States) were cut using a knife plotter (GS-24, Roland DGA, United States). Access holes of 1 mm in diameter were drilled into glass slides as an interface

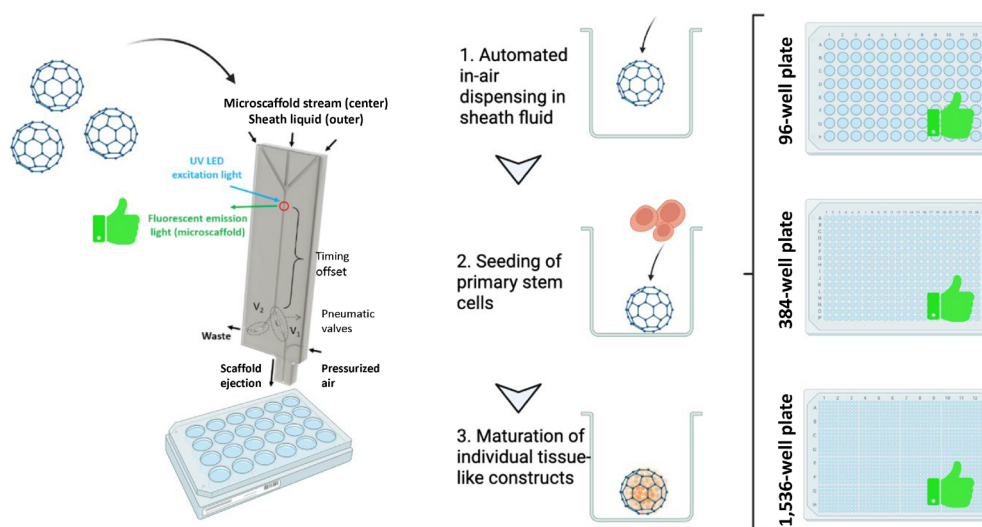


Figure 1. Schematic of a microfluidic platform for high-throughput, automated fabrication of scaffolded spheroids (S-SPH). Validated scaffolds, deposited and seeded with primary stem cells in wells, mature into S-SPH building blocks for tissue engineering applications.

Abbreviations: LED: Light-emitting diode; UV: Ultraviolet.

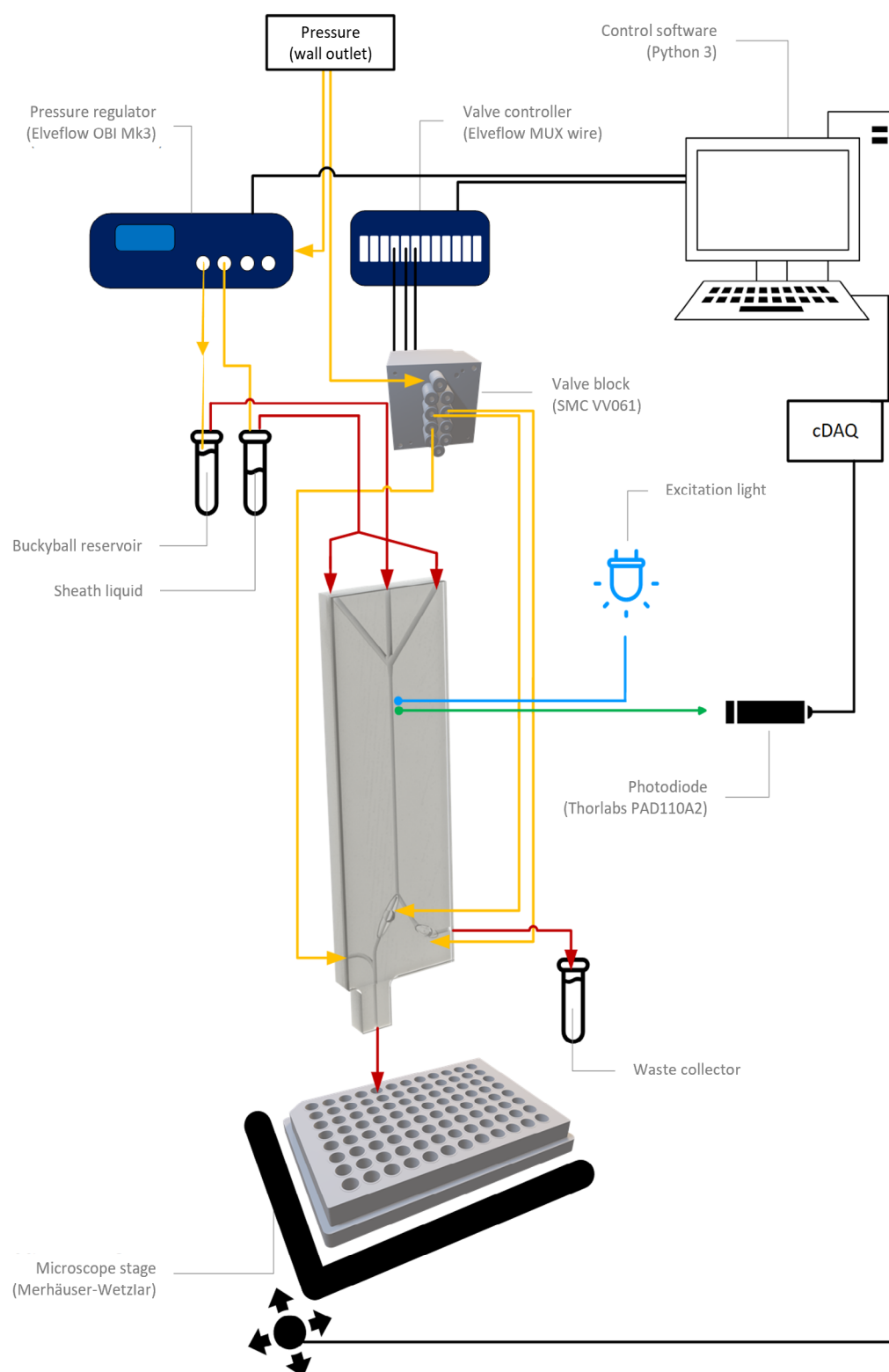


Figure 2. Schematics of the microfluidic deposition setup. Pressure regulators apply air pressure to the Falcon tubes to deliver microscaffolds and sheath liquid to the microfluidic chip. The valve block controls on-chip valves to singularize passing buckyballs based on fluorescence detected by a photodiode. The microscope stage moves the well plate from well to well. Central control was performed via the Python software.

layer and cleaned in 70% ethanol in an ultrasound bath for 15 minutes at room temperature before accommodating the pneumatic tubing. The layers were bonded by placing two sequential layers into a plasma-bonding chamber (Basic Plasma Cleaner, PDC-32G-2, Harrick-Plasma, United States) for two minutes at the highest power setting. The two layers were carefully aligned on top of each other, pressed together for five minutes, and cured in an oven at 70°C for at least one hour before adding the next layer. Once all the layers were assembled, the device was placed in the oven at 70°C overnight for a final bake to increase the bonding strength between the two types of silicone used for the production. Finally, 1 cm long pneumatic tubing was fixed on the interface using a two-component epoxy glue (LOCTITE EA 9492, Henkel, Germany) cured at 70°C for four hours to ensure air-pressure-driven actuation of the on-chip valves.

2.3. Fabrication of microscalloids

The microscalloids were fabricated using a high-resolution 3D printing method of two-photon polymerization, as previously described by Guillaume *et al.*¹⁷ Zirconium sol-gel (ZrHyb) was used as described by Ovsianikov *et al.*¹⁸ In brief, 0.5% w/w of 4,4'-bis(diethylamino)benzophenone (160326, Sigma-Aldrich, United States) was dissolved as a photoinitiator in the ZrHyb containing 1-propanol as a solvent. The microscalloids were produced using the following settings: laser power of 140 mW, scanning speed of 1,000 mm/s, a hatch distance dX/dY of 0.5 µm, and a layer spacing dZ of 2.5 µm. After structuring using two-photon polymerization, the sample holder was submerged in 1-propanol for an hour to dissolve any uncrosslinked material. Two additional washing steps were performed before storing the microscalloids in 1-propanol.

2.4. Movement tracking using TrackMate

Videos were recorded at 120 frames per second (fps) and subsequently converted to 30 fps, cropped to the area of interest, and exported as uncompressed Audio Video Interleave files (8-bit, UYUY codec) with Premiere Pro (version 2020, Adobe, United States). Movement speed of the microscalloids was tracked via the TrackMate plugin v6.0.3¹⁹ in Fiji v1.53c.²⁰ After importing, the files were converted to greyscale, and the region of interest was set around the central channel. Particles were detected using the difference-of-Gaussian detector in TrackMate with a 20-pixel radius, 2-pixel thresholds, and no filters. The quality filter was applied to remove unneeded dots until only the main track was visible. Tracks were then generated using the Laplacian filter with a maximum distance of 20 pixels and a segment gap closing of 20 pixels over five frames. Finally, filtering by track ID allowed the extraction

of the speed for each microscalloid.

2.5. Fluorescence spectrum analysis of microconstructs

The produced microscalloids were fluorescent due to the presence of the photoinitiator in the material, and were leveraged for the on-the-fly detection and analysis process. The fluorescent spectrum of BBs produced from ZrHyb was characterized by placing around 100 microscalloids into a 96-well plate in 200 µL of 1-propanol. The samples were excited at 360 nm, and their emission spectrum were analyzed on a photometer (BioTek Synergy H1, Agilent, United States) from 390 nm to 700 nm at intervals of 10 nm. The excitation wavelength was determined from literature values stating that the photoinitiator showed the highest absorption potential at 365 nm.²¹ The emission values were subtracted from the background signal emitted from the well plate and the 1-propanol.

2.6. Optical detection device

The optical detection mechanism was built using an excitation light-emitting diode (LED) with a wavelength of 360–370 nm (3W370380m, Avonec GmbH, Germany). The LED was powered with a constant current source (LDD-700LW, Mean Well, Taiwan) at 100% output. The light source was collimated and focused using a condenser lens (ACL25416U, Thorlabs, United States). Light splitting was performed via a dichroic mirror (DMLP490R, Thorlabs, United States) housed in a metric filter mount (CM1-DCH/M, Thorlabs, United States). To distinguish between excitation and emission wavelength, a 500 nm long-pass filter (62983, Edmund Optics, United States) and a short-pass filter at 475 nm (84705, Edmund Optics, United States) were used. The LED light was positioned at a precise distance from the lens using a 3D-printed holder. The fluorescent excitation signal was detected using a photodiode (PDA100A2, Thorlabs, United States). The signal was forwarded to a voltage input module (NI-9215, National Instruments, United States) housed in a compact data acquisition chassis (cDAQ 9171, National Instruments, United States). The device was queried by the NI-DAQmx library (version 20.0)²² within a custom-written Python 3 program.

2.7. Production and culture of scaffolded spheroids

The 96-, 384-, and 1,536-well plates were pre-filled with 200, 20, and 10 µL of deionized water, respectively, using the automatic dispensing function in the controlling program. Microscalloids were then dispensed into each well. The plate was then centrifuged for two minutes at 200 × g to position the BBs to the center of the wells in the plate. Water was evaporated at 60°C on a heated plate for

several hours. The well plates were then sterilized using the ultraviolet lamp in a laminar flow cabinet. The microfluidic sorting chip was used to dispense 200, 20, or 10 μL of cell-laden medium from a stock solution into each device. The stock solution contained immortalized hASCs (Evercyte, Austria), which were expanded using EGM™-2 BulletKit™ medium (Lonza, Switzerland) supplemented with 10% (v/v) newborn calf serum (Gibco, New Zealand) and maintained at standard culturing conditions (37°C, 5% carbon dioxide, humidified atmosphere). The concentration of the stock was adjusted to deliver either 1,000 or 5,000 cells in the required working volume of the well plate. Spheroids were maintained for 12 days, with the culture media changed every 2–3 days. The spheroids were imaged at 24 hours and 11 days using an optical microscope (BioTek Synergy H1 microplate reader, BioTek, United States) to assess spheroid formation.

2.8. Metabolic activity analysis

Metabolic activity was evaluated using the PrestoBlue assay (A13261, Invitrogen, United States). After depositing cells, either automatically or manually, 10 μL of the agent was added to the existing 90 μL of culture volume and allowed to react for 24 hours under standard culture conditions. Fluorescence was analyzed via a plate reader (BioTek, United States) at an excitation of 570 nm and an emission of 590 nm. The fluorescence was normalized to the actual number of seeded cells derived from a diluted standard.

2.9. Statistical analysis

An unpaired *t*-test (R, version 4.0.0) was performed to assess significance, with *p*-values <0.05 and <0.01 considered significant, without applying any corrections.

3. Results

3.1. Sorting device design and characterization

Figure 3A shows the design of the microfluidic device that was used to sort microscaffolds into individual cell culture wells. The device consisted of four parts: a bottom PDMS layer containing the fluidic channels, a deflective PDMS membrane to block the flow, a pneumatic PDMS layer, and a glass layer, as shown in Figure 3B. The latter three layers formed on-chip valves (V_1 and V_2) actuated via air pressure. The different stages of the sorting procedure are illustrated in Figure 3C. The microscaffolds were delivered to the microfluidic device using polytetrafluoroethylene tubing with an inner diameter of 0.8 mm. Polytetrafluoroethylene was selected over silicon, as using silicon would cause the BBs to get stuck near the opening at the uptake. Furthermore, an inner diameter of 0.8 mm was selected to restrict the microscaffolds from entangling during delivery to the sorting device. Microscaffolds entered the chip via the inlet channel in the middle (t_0 , Figure 3C). Sheath liquid from the adjacent channels confined the microscaffolds to the center of the stream, singularizing them. After

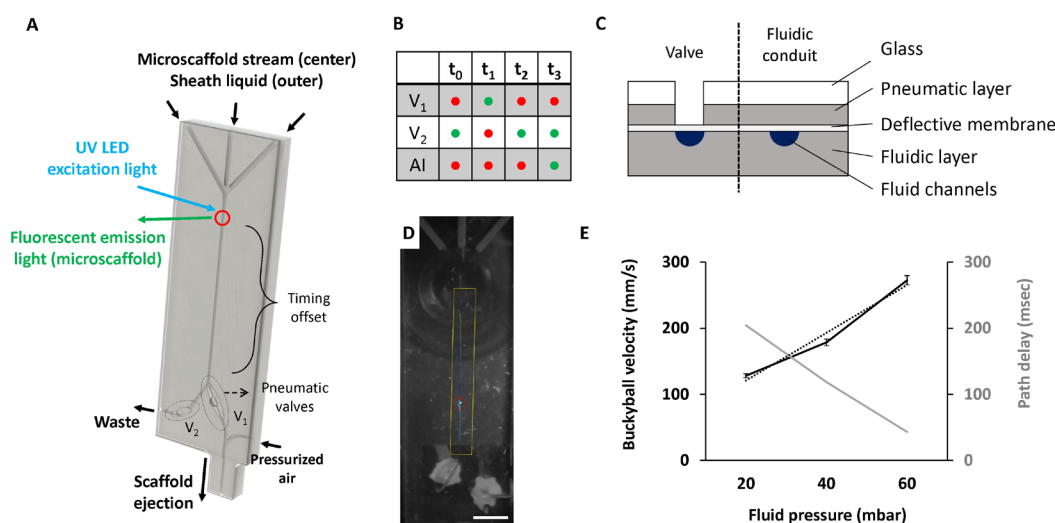


Figure 3. Design and functionality of the microfluidic sorting device. (A) The microfluidic device guides microscaffolds past the detection area to either sort buckyballs in the well plate beneath them or direct them toward the waste outlet. (B) Cross-view of pneumatic valves and fluidic conduit of the sorting device. (C) Sequence overview of sorting procedure. V_1 and V_2 denote on-chip valves, and AI denotes pressurized air inlet. Colors reflect their state (green = open, red = closed) at different sorting stages. Abbreviations: LED: Light-emitting diode; UV: Ultraviolet.

passing an optical detection area, BBs were either directed toward the waste channel or the sorting channel (t_1) by manipulating the on-chip valves downstream. Once microscaffolds were directed into the ejection channel, the pneumatic valves sealed this channel from the main stream (t_2) to evacuate its content downward using pressurized air (t_3). Microscaffolds were then ejected through a metal nozzle (not shown) into a tissue culture well underneath. The different stages of the sorting procedure are shown in Figure 3B. Furthermore, a cross-section of the on-chip valves and the fluidic conduits is illustrated in Figure 3C. The on-chip valves were actuated via pressurized air inlets, deflecting the thin PDMS membrane.

Flow focusing was used to arrange the incoming microscaffolds in succession, generating single events at the downstream fluorescent detection area. With microscaffolds measuring 300 μm in diameter, a sample stream width of around 400 μm was targeted. As seen in Figure 4A–F, the width of the central sample stream changed by varying the liquid pressure ratio between the sample and sheath liquid. At a 1:1 ratio, the inner channel took up 50% of the channel ($\sim 400 \mu\text{m}$). At a 1:2 ratio, the inner channel was reduced to 30% ($\sim 240 \mu\text{m}$), and at a 1:3 ratio, it was further reduced to 20% ($\sim 165 \mu\text{m}$). Conversely, at a ratio of 2:1, the channel took up 71% ($\sim 570 \mu\text{m}$). At a ratio of 3:1, no further distinction between sample liquid and sheath liquid could be made, similar to when sheath flow was turned off completely, as quantified

and summarized in Figure 4J. The laminar flow regime in the sorting device was kept intact regardless of the chosen pressure ratios (Figure 4A–F) and flow speeds (Figure 4G–I).

Once the microscaffolds flowed in succession, their travel speed was measured to determine the timing offset between BB detection and rerouting back to the ejection channel (Figure 3A). For this, videos of the microscaffolds were recorded at 20, 40, and 60 mbar fluid pressure and evaluated using the TrackMate19 plugin within Fiji (Figure 4K). The single tracks were then analyzed to derive a mean microscaffold velocity in mm/s and approximated via linear regression using the formula $y = 3.614x + 48.569$, where x denotes the fluid pressure in mbar and y the resulting microscaffold velocity in mm/s (Figure 4L). The error bars showed a flow speed standard deviation of 3%, 5% and 7% at 20 mbar, 40 mbar, and 60 mbar, respectively. Timing offsets between BB detection and channel seclusion of 304 ms, 218 ms, and 143 ms were calculated for fluid pressures of 20, 40, and 60 mbar, respectively. As the on-chip valves required a response time of around 100 ms to fully react to the electronic signal, the timing offsets were reduced by 100 ms, leading to the final values of 204 ms, 118 ms, and 43 ms for the three fluid pressures, respectively.

A timing offset of 200 ms at a fluid pressure of 20 mbar was also determined experimentally (by reducing the offset until BBs could be sorted repeatably), thereby confirming the results derived from the particle tracking.

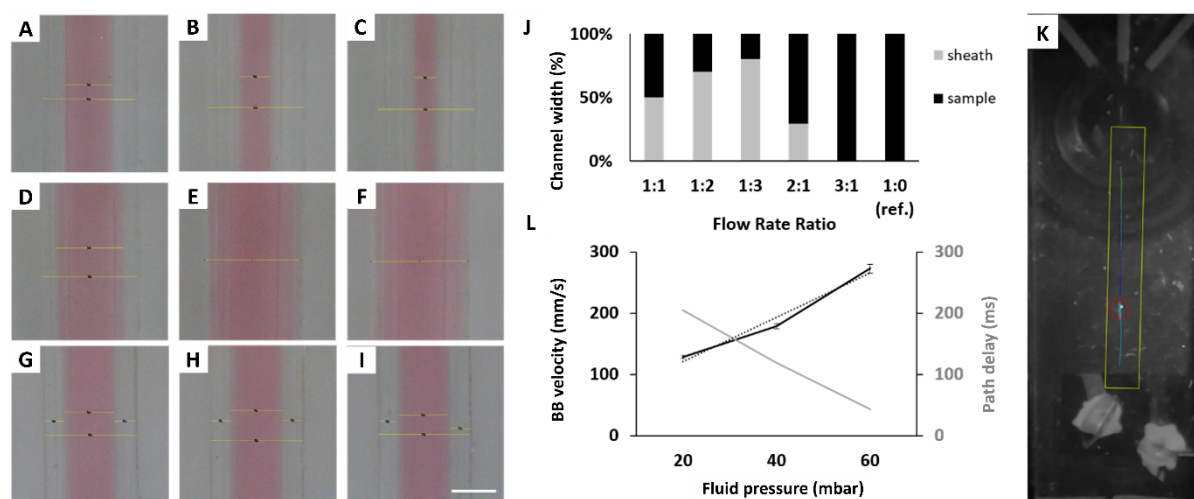


Figure 4. Characterization of flow focusing ratios and buckyball (BB) movement speeds in the sorting device. Influence of flow focusing ratios (pressure ratio of the sample fluid and the sheath fluid) on the width of the central sample stream (red) at a ratio of (A) 1:1, (B) 1:2, (C) 1:3, (D) 2:1, (E) 3:1, and (F) 1:0. A 1:1 ratio yielded a sample stream width of 400 μm regardless of the fluid pressure, demonstrated at (G) 30 mbar, (H) 40 mbar, and (I) 50 mbar. Scale bar: 400 μm . (J) Quantification of stream widths at varying flow rate ratios. (K) The traveling trajectory of BBs is tracked via TrackMate, with the microscaffold in flow circled in red, the region of interest in yellow, and the trajectory in blue. (L) Travelling speeds of BBs quantified at 20 mbar, 40 mbar, and 60 mbar.

The timing offsets were described as a linear function of the fluid pressure and were approximated by the formula via linear regression, $y = -4.035x + 283.341$, where x is the fluid pressure in mbar and y is the path delay before switching the valve in ms. Linear regression was chosen as a linear correlation between particle velocity and sheath flow velocity was reported in the literature²³. The BB velocity and the respective timing offsets are illustrated in Figure 4L.

3.2. Optical detection system

A detection system was built to identify the single BBs in flow, based on the fluorescent properties of the photoinitiator 4,4'-bis(diethylamino)benzophenone in the ZrHyb microscaffolds. The optical detection system for in-line detection of BBs in flow was built as shown in Figure 5A, following the fundamental optical setup of a widefield fluorescence microscope. The fluorescent emission spectrum of the microscaffolds produced from ZrHyb was first determined to optimize the wavelength used in the

optical detection system. The absorption maximum of the photoinitiator 4,4'-bis(diethylamino)benzophenone was previously reported to be at 360 nm²¹. At an excitation of 360 nm, BBs in 1-propanol showed maximum emission at 530 nm (Figure 5B). Therefore, an LED with a wavelength of 370–380 nm was used to excite passing microscaffolds. Passing microscaffolds emitted light at a wavelength of 530 nm as a response to the excitation light, which was sensed by the photodiode and was registered in the main control program as a short-term increase in voltage. This change in voltage was then used to differentiate between the empty channel with carrier liquid (background signal) and an intact microscaffold (peak signal). Signals between the two values were further interpreted as scaffolds with structural defects or debris, depending on the intensity of the signal. Thresholds for these signals were determined experimentally and are shown in Figure 5C. First, the background signal by the LED was determined to be around 262 ± 1 mV. After the addition of 1-propanol and the use of the sorting device, the background signal

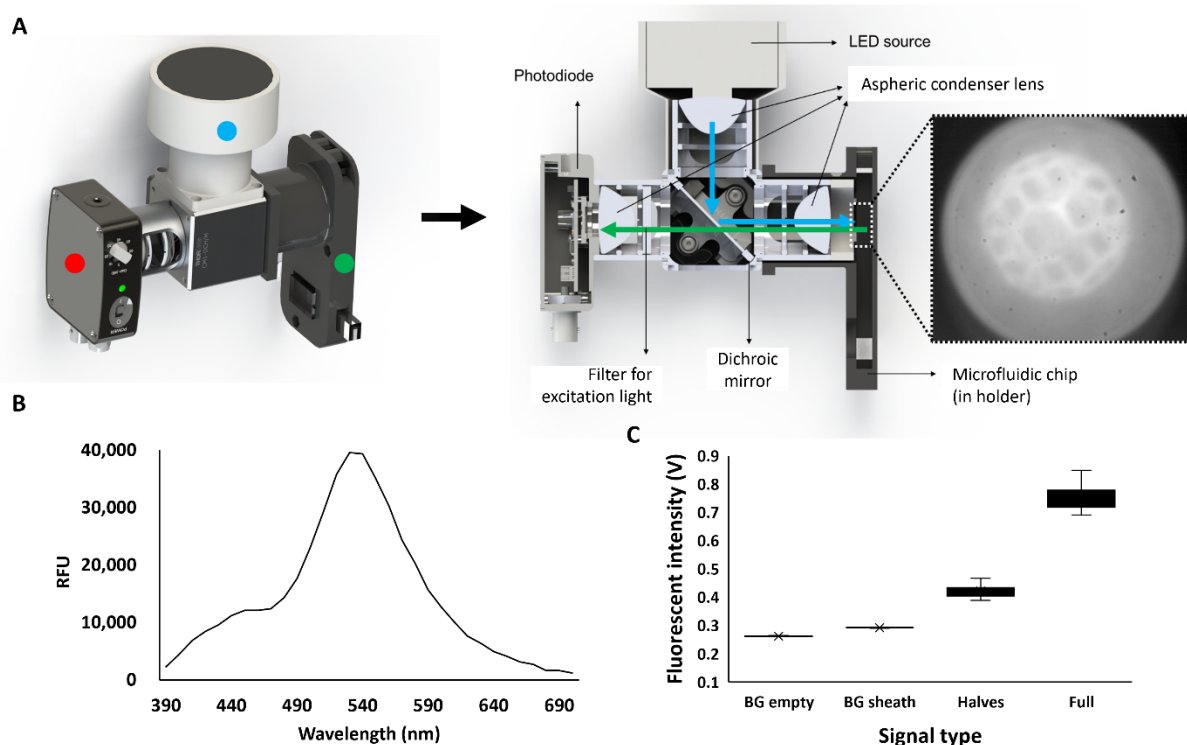


Figure 5. Optical detection setup. (A) Computer-aided design of the fluorescent detection setup. Blue dot = light source, green dot = holder + microfluidic device, red dot = photodiode. Cross-section detailing the optical path of excitation (blue) and emission (green) light, and view of the passing microscaffold as seen by the photodiode (when replaced with a camera system). Scale bar = 100 μ m. (B) Fluorescent emission spectrum of microscaffolds produced from ZrHyb at an excitation wavelength of 360 nm. (C) Light intensity measured using a photodiode. “BG empty” is the background signal of the optical device, “BG sheath” is the background signal when the PDMS device was added and flushed with sheath liquid only, “Halves” denotes microscaffold-halves used to emulate debris, and “Full” denotes intact BBs.

Abbreviations: LED: Light-emitting diode; PDMS: Polydimethylsiloxane; RFU: Relative fluorescence unit; ZrHyb: Zirconium sol-gel.

slightly increased to 292.33 ± 1 mV. A batch of intact microscaffolds resulted in a mean detection value of 749 ± 59 mV. In comparison, a batch of half-BBs was printed to simulate manufacturing defects, which showed a mean detection value of 422 ± 24 mV (Figure 5).

Following these results, 0.70 V was used as a threshold value for the sorting algorithm to identify a passing scaffold as intact. Furthermore, values above 0.4 V were used as thresholds to identify “debris” in the fluidic channel. Values below 0.4 V were ignored as background signals. These values were used as thresholds for sorting BBs in all subsequent experiments.

3.3. Sorting process of buckyball microscaffolds

Two values were manipulated to sort the microscaffolds. First, the fluorescent intensity of an event determines the quality of the BB, and second, the timing offset between two events determines whether they would interfere with each other's sorting process. The underlying logic is visualized in Figure 6. The incoming data stream from the photodiode was supervised for intensity threshold crossings. Threshold upward and downward crossings signaled a BB entering or leaving the detection area on the chip, respectively. These events were then flagged as sortable or not sortable, depending on their time offset to other events and their peak fluorescence intensity.

Upon upward crossings of the debris threshold, the time difference to the previous event's (e_1) downward crossing time-point was measured (Δt). If the new event e_2 was too close to e_1 , e_2 was flagged as non-sortable (red). As e_2 would further impact the sorting process of the previously approved event e_1 , its sorting process had to be aborted retroactively. For this purpose, an additional event parameter was defined and checked by the sorting algorithm immediately before directing the BB into the ejection channel. If there was enough distance between events, e_2 was pre-flagged as sortable (orange), and e_1 received its final sorting flag (green), allowing it to be sorted.

When the signal crossed the threshold downward, the algorithm checked whether the current event (e_2) was flagged as sortable or non-sortable (orange or red, respectively). Events flagged as non-sortable were ignored. For events pre-flagged as sortable (orange), the peak fluorescent value was evaluated. If the value surpassed the fluorescent threshold of 0.7 V, e_2 was categorized as intact BB and ultimately flagged as sortable (green). Otherwise, e_2 was categorized as debris and ultimately flagged as non-sortable (red). Lastly, a new timestamp was set to calculate timing offsets for future events (e_3).

The values for classifying microscaffolds as either intact or debris were derived from Figure 5C, whereas the minimum distance between microscaffolds was calculated as the sum of events within the sorting cascade without the time required to move the microscope stage between the wells. For a liquid pressure of 20 mbar, this time was determined to be 900 ms; therefore, a time threshold of 1 second between events was chosen.

Once microscaffolds were flagged sortable (green), a sorting sequence was started. This sequence consisted of an initial waiting period that would allow the BB to arrive at the bifurcation (values chosen from Figure 4L). Just before actuating the valve, the flag of the BB event was checked one last time. This double-flagging system was introduced to abort sorting events at the last moment, due to a subsequent BB following too closely. This double-flagging greatly reduced the amount of microscaffolds stuck in the PDMS valve. After a BB was secluded in the ejection channel, it was dispensed into a cell culture well plate beneath using pressurized air. Repeatable droplet ejection was possible at 60 mbar for 500 ms. Shorter ejection times did not fully clear the channel, whereas higher pressures resulted in sideways spraying of the droplet.

The sorting efficiency of the system was determined as a percentage of wells containing exactly one microscaffold, which amounted to 95%, measured on a total of 250 microscaffolds. The system's capability to effectively distinguish between intact microscaffolds and debris was assessed by sorting a mixed sample containing intact BBs and half-BB simulating debris. Next, a sample size of 96 sorting events emulating the production of a fully loaded 96-well microtiter plate was analyzed, with only intact microscaffolds being dispensed, leading to 100% sorting efficiency. Once the sorting efficiency was determined for 96-well plates, 384-well plates, and 1,536-well plates, they were sorted to increase the quantity of sorted microscaffolds while maintaining the same plate form factor (Figure 7A). The time taken from event detection to scaffold ejection and moving to the next microwell was 2.5 seconds. Depending on the microscaffold concentration in the reservoir, sorting a full 96-well plate typically took between 12 and 15 minutes, whereas 384-well plates required between 50 and 60 minutes. Microscaffold reservoirs were loaded at 400 BB per 50 mL of liquid. An even distribution of the BBs in the reservoir was crucial to keep the concentration stable. This was especially important when sorting larger well plates, such as 384-well plates, which required re-stocking of the reservoir mid-sort.

3.4. Pilot study on the generation of stem cell spheroid culture within buckyball microscaffolds

In a final proof-of-concept study, the successful deposition

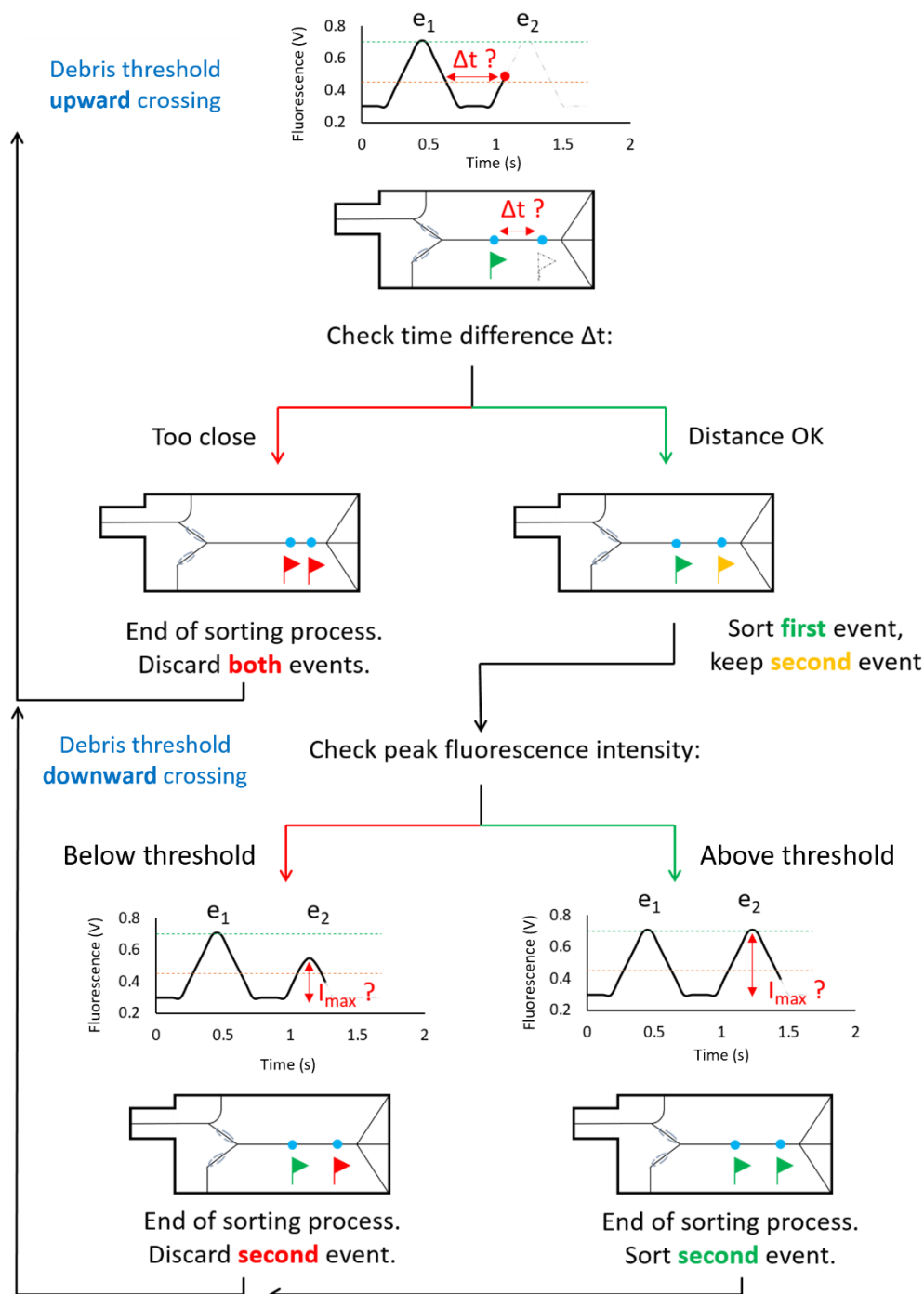


Figure 6. Flow diagram for double-flagging of events shown via the data stream captured by the photodiode and the on-chip events. The process is initiated upon the upward crossing of the debris threshold value. The time difference to a previous event is evaluated. If an earlier event is too close, both events are flagged as non-sortable (red flag). If the distance is above the distance threshold, the first event is sorted (green flag), whereas the following event is pre-flagged for sorting (orange flag). Upon the downward crossing of the fluorescent intensity, the peak intensity value is validated. Depending on whether it is below or above a threshold, the event is flagged for sorting (green flag) or discarded (red flag).

of microscaffolds in culture wells by our optimized microfluidic sorting protocol was applied by deposition of hASCs in culture medium to form S-SPHs. Cell deposition was executed via a separate fluid line to minimize the number of wasted cells and improve repeatability compared to utilizing the microfluidic device for this task (Figure S1). The repeatability was measured at a constant fluid pressure of 50 mbar and a valve opening time of 500 ms and 1,800 ms, for which the fluid line showed a dispensed volume of $66.6 \pm 7 \mu\text{L}$ and $245.4 \pm 1.8 \mu\text{L}$, respectively

(Figure S1B). Next, the impact of automated deposition on the metabolic activity of hASCs was assessed. hASCs were dispensed automatically and manually, and their metabolic activity, expressed as relative fluorescence units, was compared after 24 hours of culture via the PrestoBlue assay. The fluorescence was normalized to the actual number of deposited cells derived from a diluted standard (Figure 7C). Overall, cells deposited automatically showed an 8% lower metabolic activity after 24 hours of culture compared to cells that were dispensed manually. To

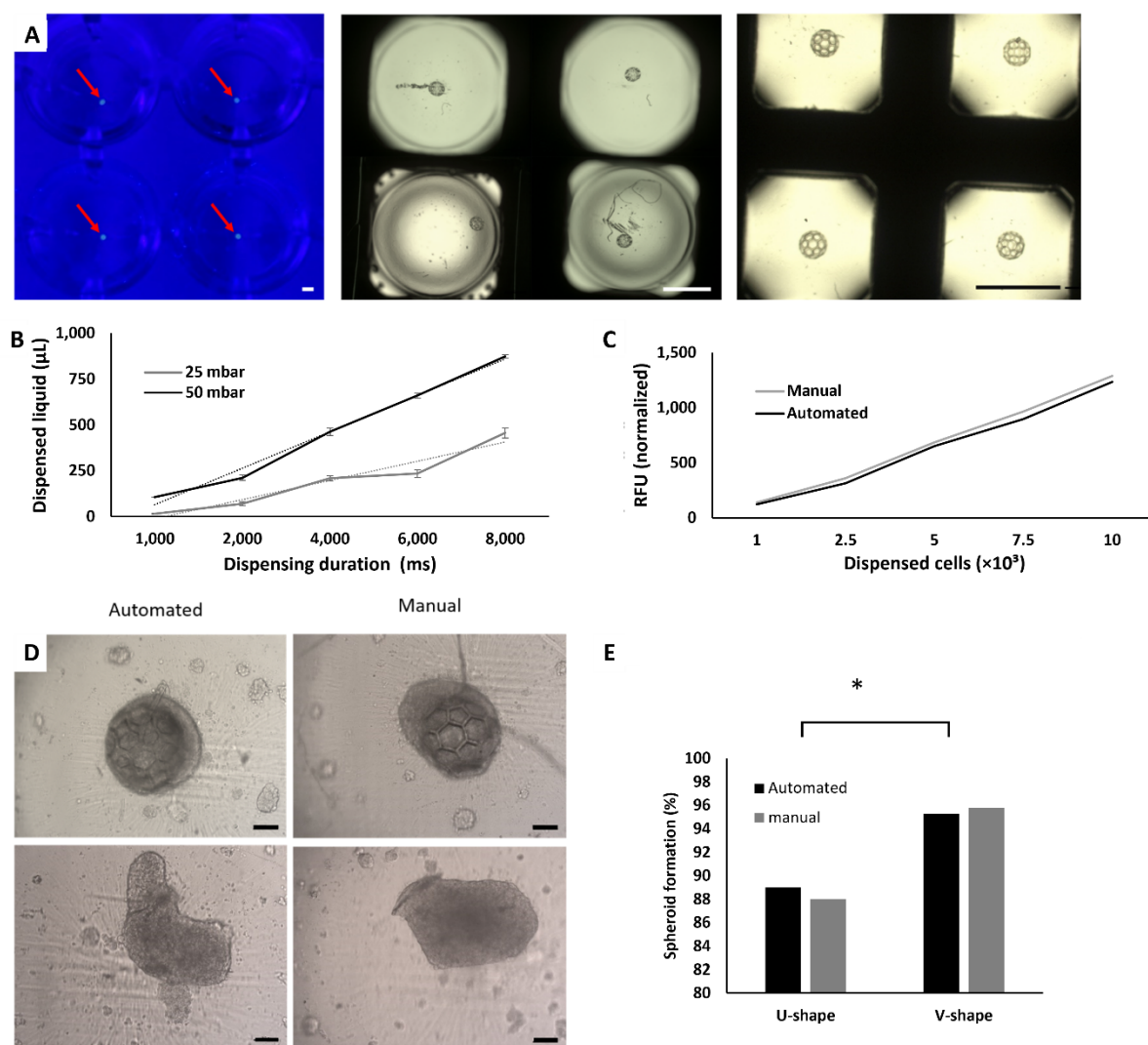


Figure 7. Automated deposition of microscaffolds and hASCs to form scaffolded spheroids (S-SPHs). (A) Microscaffolds sorted into well plates of different well numbers (96-, 384-, and 1,536-well plates; red arrow denotes buckyballs). Scale bar: 1 mm; magnification: 4×. The 384-well plate image is a merge of four separately taken images. (B) Correlation of valve opening times and dispensed cell volume at 25 and 50 mbar. (C) Metabolic activity of automatically and manually dispensed hASCs. (D) Spheroid formation in a 384-well plate after 24 hours, comparing automatic and manual cell deposition with and without microscaffold. Scale bar: 100 μm ; magnification: 10×. (E) Percentage of spheroid formation comparing automated and manual deposition modes in U-shaped and V-shaped well plates. * indicates significance at $p < 0.01$.

Abbreviations: hASCs: Human adipose-derived stem cells; RFU: Relative fluorescence unit.

automate the production of S-SPHs, the sorting step and the dispensing step were combined. Initial tests showed that only microscaffolds that were placed in the center of the well would instigate the formation of round spheroids within. Those placed off-center would either not receive enough cells to form a spheroid or form an elongated cell cluster outside of the BB. To account for this, wells were pre-filled with 200 μL of deionized water, and an intermediary centrifugation step was added between sorting and seeding the microscaffolds. Furthermore, the correct choice of a well plate was crucial to the central placement of the BB and, therefore, the formation of spheroids. Only plates with a V-shaped bottom were able to position the BB in the exact center after centrifugation. U-shaped plate bottoms merely positioned BBs close to the center, whereas flat-bottom plates proved completely inept. Spheroid-laden microscaffolds were formed after 24 hours of culture using 5,000 hASCs. Twenty-four hours after seeding the microscaffolds, 95% of the microscaffolds showed the formation of a spheroid in a V-shaped 96-well plate, regardless of automatic or manual dispensing (Figure 7D). In U-shaped 384-well plates, an overall higher percentage of spheroid non-formation was visible, with automatic deposition showing 11% non-formation, whereas manual deposition resulted in 12% of the wells not forming a spheroid. While there was no significant difference between these groups (p -value = 0.916), the type of bottom shape had a significant influence (p -value = 0.0016) on the formation of a spheroid (Figure 7E). Seven days after the initial seeding, the number of formed spheroids had remained unchanged, indicating that if no spheroid formation had occurred within 24 hours, it would no longer appear at a later stage.

4. Discussion

Unlike state-of-the-art microfluidic flow cytometers, the device presented here was developed to eject microscaffolds and liquids after the sorting process, which imposed certain design considerations on the microfluidic device. Typically, microfluidic sorting devices continuously channel their content into predefined integrated channels within the device. In contrast, our device necessitated relocating content outside the device to a precise location. To address this requirement, we developed a system with a temporal interruption, enabling content seclusion and ejection without disrupting the flow of the main inlet stream. Additionally, the synchronization of the ejection process with the availability of an empty well further justified our departure from a continuous processing approach. A dedicated fluid line was added to automate cell deposition on top of the microscaffolds and produce spheroid-laden microscaffolds in a scalable manner. Sample focusing was

achieved via sheath flow, which precisely controlled the central sample stream width, as seen in Figure 4A–I. In comparison to flow focusing via dielectrophoresis²⁴ and acoustic force,²⁵ this method maintains a stable pH and is independent of the to-be-focused particle size.

To isolate a single particle and eject it into a single culture well, the developed system splits the sorting process into secluding and subsequently ejecting. Both methods were combined into one functional element by implementing pneumatic on-chip valves that physically separate the ejection channel from the main channel.

By combining fluorescent intensity and distance between events as metrics with the approach of double-flagging, this staged sorting process enabled the highest sorting yield, defined by the number of wells per plate containing a single microscaffold. However, this comes at the expense of a higher rate of rejected microscaffolds and other challenges, such as BBs trapped in the valve—leading to an empty well—and BBs crushed by valves—leading to debris dispensing in the system.

While deposition with the chip proved to be precise, switching to a dedicated line for cell delivery caused less stress on the microfluidic device. Given its lengthy fabrication process, preserving its functionality was a clear benefit. Furthermore, the dedicated line allowed a higher fluid pressure and a higher dispensing throughput (Figure S1). Given that liquid dispensing was required twice per culture well—once for pre-filling and once for cell delivery—the speed of liquid delivery was a significant factor in liquid dispensing.

Moreover, using the microfluidic device for dispensing would have resulted in a wasted volume of 20 mL of medium containing 4×10^6 cells for a 384-well plate, as cell-laden medium would constantly flow to the waste channel to keep a constant flow rate while the dispensing step is in progress. For the 1,536-well plates, however, cell delivery via a dedicated line proved to be challenging. First, the well dimensions were $1.7 \times 1.7 \text{ mm}^2$, requiring the delivery line with an outer diameter of 1.6 mm to be perfectly centered. While this was alleviated by adding a dispensing tip to the end of the tubing, dislodging the droplet from the needle proved to be more problematic, as the working volume of a 1,536-well plate was specified with 4–12 μL . Initially, the needle was kept so close to the well-plate that moving to the next well wiped the tip. However, as the tip was not always positioned at the same height, it would get entangled when moving the well plate, resulting in double dispensing and overflowing wells. In addition to overflowing wells, wiping the nozzle between wells increased the risk of infection. For the 1,536-well plates, cell deposition using the microfluidic device was therefore

avored, as having an air-pressurized outlet allowed for the precise ejecting of even small volumes—down to 10 μL —repeatedly.

Excluding drying and ultraviolet sterilization, the automated workflow for preparing a fully seeded plate required approximately 20–25 minutes for 96 wells, one hour and 15 minutes for 384 wells, and about five hours for 1,536 wells. However, manual steps still added substantial time to the overall process, particularly the drying step, which took several hours. This step could be significantly shortened in the future by replacing heat evaporation with direct liquid removal or vacuum-assisted drying. Additionally, parallelization of plate handling or the use of alternative carrier liquids such as alcohols, which evaporate faster and maintain sterile conditions, could have further reduced preparation time. Notably, microscaffolds could have been presorted and stored in the liquid, allowing the cell culture workflow to start when needed. Importantly, the design of the device enabled a clear path toward scalability, as it can multiplex multiple units in parallel to easily boost the throughput, making this approach highly adaptable for future high-throughput bioprinting workflows.

As expected, the cellular metabolic activity decreased in the automatically deposited cells due to the exposure to high shear stresses in comparison to the manually deposited ones (Figure 7C). While the dispensing process shown here was not directly comparable to fluorescence-activated cell sorting, other studies have shown that depositing cells using fluorescence-activated cell sorting showed a similar negative influence on their viability and proliferation,²⁶ most likely caused by cleavage of membrane proteins that triggered further stress-related pathways.²⁷

As shown in Figure 7D, the type of well plate had an important influence on the formation of spheroids within microscaffolds. High-density well plates with a V-shaped bottom and a low-retention coating would provide the perfect culture conditions. However, the latter two parameters are typically exclusive features on commercially available well plates. Therefore, low-retention coatings were manually applied on V-shaped plates to increase the formation of spheroids.

Unlike hydrogel-based microparticles, which often suffer from low mechanical strength and deformation under shear stress, S-SPHs combine high cell density and natural cell–cell interactions with a rigid, porous microscaffold that provides mechanical stability, protection, and tunable properties, enabling their use as modular building blocks for larger tissues.

5. Conclusion

The third strategy for TE encompasses the formation of a

spheroid within a highly porous microscaffold that shields the spheroid from mechanical impact, while enabling its fusion with surrounding S-SPHs, precisely controlling the formation of individual building blocks for the bottom-up fabrication of larger tissue blocks that better resemble actual human tissues. While bottom-up TE has been discussed elsewhere,¹⁷ this work focused on addressing the limitations arising when fabricating large numbers of such building blocks for technological scale-up and mass production.²⁸ Automating particle analysis, sorting, and dispensing steps supported by the interplay of microfluidics for fluid transportation and actuation, as well as optical elements for particle characterization, was successfully optimized. We have shown a device capable of producing a 95% sorting yield when singularizing microscaffolds at 100% precision when the sorting solution was challenged with 50% defective constructs. The precision of the system enabled not only the automation of scale-up production of BBs across various cell culture well plates, but also automated cell deposition and subsequent spheroid growth within microscaffolds of up to 1,536 wells, ensuring high throughput building block formation within 24 hours.

Overall, the results of our study could advance the production of previously optimized hybrid spheroid microscaffolds as modular tissue building blocks,¹⁷ providing a robust basis for production pipelines that support the third strategy in TE.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Data will be made available upon request to the corresponding author.

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